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14. ABSTRACT

Gene therapy shows promise for treating prostate cancer and is being exploited in several clinical trials. A major hurdle is establishing a method of verifying transgene activity *in situ*. β-galactosidase (β-gal) was historically the most popular reporter gene for molecular biology. I have introduced a novel concept for further exploration of gene therapy using β-galactosidase to activate a broad-spectrum chemotherapeutic to assess the efficacy of the pro-drugs *in vitro* and explore growth delay in animal models. I also have developed a new β-galactosidase molecular reporter for MRI spectra, which can be used to detection of lacZ gene expression in vivo.

15. SUBJECT TERMS
Prostate cancer, β-galactosidase, Gene therapy, PFONPG

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Introduction

Prostate cancer is one of the most common malignant tumors with increasing incidence rates in the aging male, presenting a formidable public health problem. Gene-based therapy has been stimulated by remarkable progress in understanding molecular biology. Gene therapy holds great promise for the treatment of diverse diseases. The lacZ gene, encoding the enzyme β-galactosidase (β-gal), has historically been the most common reporter gene used in molecular biology, many chromogenic or fluorogenic substrates are well established, but they are generally limited to histology or *in vitro* assays. Our prototype molecule PFONPG (para- fluoro- ortho-nitro- phenyl β-D-galactopyranoside) is a direct analog of the traditional "yellow' biochemical indicator ONPG (ortho- nitro- phenyl β-D-galactopyranoside). Our research team applied PFONPG for gene therapy and developed new spectral reporter molecules to assess gene expression in vivo. I also developed lacZ and luc, lacZ and GFP double gene transfected prostate cancer cell lines to detect gene activity, in particular to test tumor growth and potentially specific cytotoxic agents in cultured prostate cancer cells and reveal β-gal activity *in vivo* in transfected prostate tumors in mice.

Body

Task 1. Develop a series of expression vectors for expressing LacZ and LacZ/LacY fusion open reading frame (Completed Year 1)

I successfully excised the whole lacZ gene fragment including lacY from the expression vector pSV-β-galactosidase (Promega, MI) using the unique restriction sites BamHI and HindIII and inserted into high expression mammary expression vectors phCMV and pcDNA3.1 to yield phCMV/lacZ and pcDNA3.1/lacZ(fig.1a and 1b).

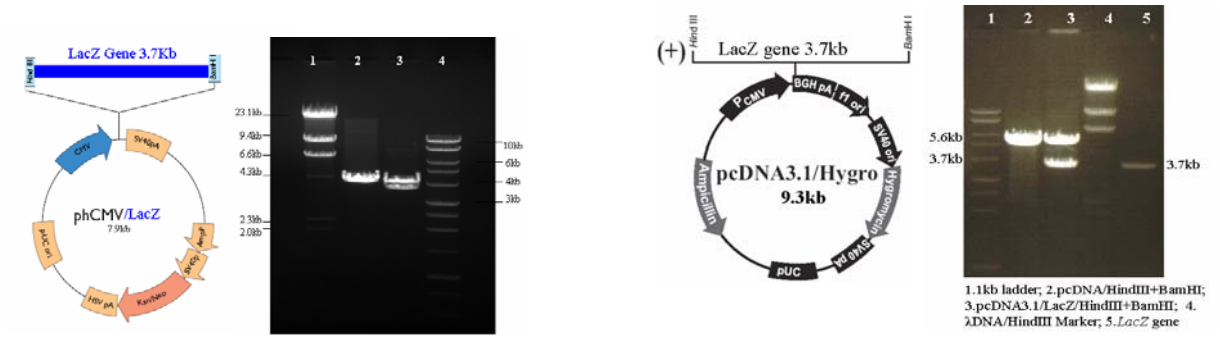


Fig 1. Recombinant vector of LacZ map and DNA separated in agrose gel (a) phCMV/lacZ; (b) pcDNA/lacZ

In order to detect lacZ expression in the recombinant vector, we transformed the phCMV/lacZ vector to *E.coli* DH5a, and then selected different colonies to inoculate the Lysogeny Broth (LB) plate with X-gal (blue), S-gal (black), AZD-3 and AZD-5, comparing *E.coli* DH5a with the empty vector phCMV as control. LacZ expression was readily detected with each agent demonstrating the correct open reading frame lacZ and lacY fusion gene.

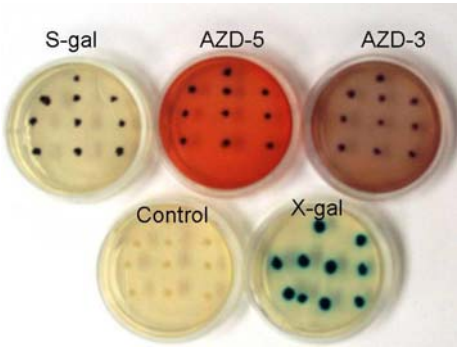


Fig 2. phCMV/lacZ recombinant vector in *E.coli* DH5a

Task 2. Transfect, screen and evaluate transfection efficiency and the optimal cell colonies for expressing β-galactosidase and lactose permease in CaP cells (Completed Year 1)

Prostate cancer cells including MAT-Lu and PC3 were cultured at 37°C in a 5.0% CO_2 atmosphere. The PC3 cells were transfected using GenerPORTER2 (Gene Therapy Systems) under control of the high expression human cytomegalovirus (CMV) immediate-early enhancer/promoter vector phCMV/lacZ. Colonies selection was applied to identify those PC3 cells with the highest β-gal expression (Fig. 3). I selected the highest expression lacZ PC3 cell clone 12 named PC3-lacZ cell. PC3-lacZ cell was stained with X-gal, S-gal, ADZ-3 and AZD-5 (Fig. 4), more than 90% PC3-lacZ cells showed high expression β-gal (Passage 15).

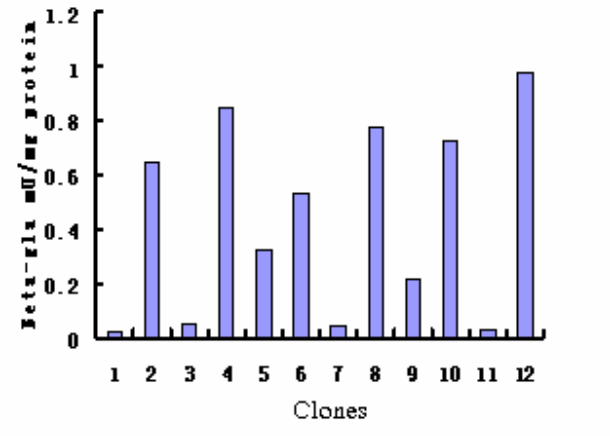


Fig 3.Detection of lacZ expression in PC3 transfected cell

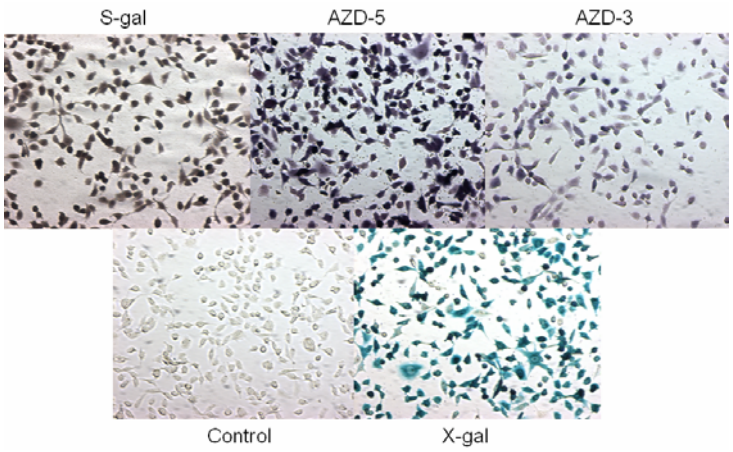


Fig 4.Detection of lacZ expression in PC3-lacZ and PC3-Empty vector cells

Task 3. Test lactose permease function in the transfected prostate cells

Since I transfected cells with the dual expression cassette of both lacZ and lacY, I assured that detected of beta-gal amount both genes were active. I did not test lactose permease function in the transfected prostate cells although it may be important to do so in the future.

Task 4. Evaluate growth of cells *in vitro* and *in vivo* (Completed Year 2)

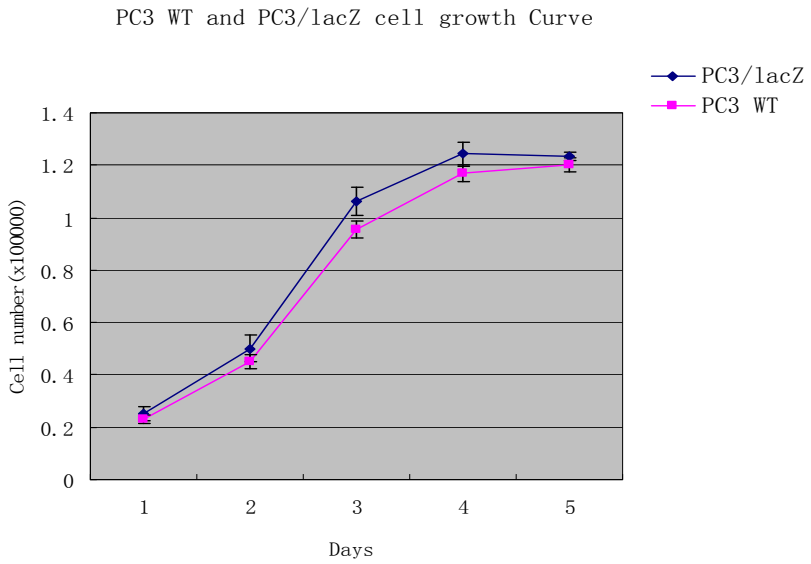


Fig.5. PC3 and PC3-lacZ cells growth curve

PC3 WT and PC3-lacZ cells ($3x10^4$) were seeded into 24-well plates, the cell number in three to five flasks was determined at different times. PC3-lacZ expressing clones had slightly faster growth rate the parental cells (WT).

For in vivo study, $2X10^6$ PC3 and PC3-lacZ cells were implanted subcutaneously into male nude mice. Tumor growth was followed. Palpable tumors were formed 7-10 days after the injection. The lacZ over expressing cell lines formed tumors the grew much faster than the parental WT. (fig.6)

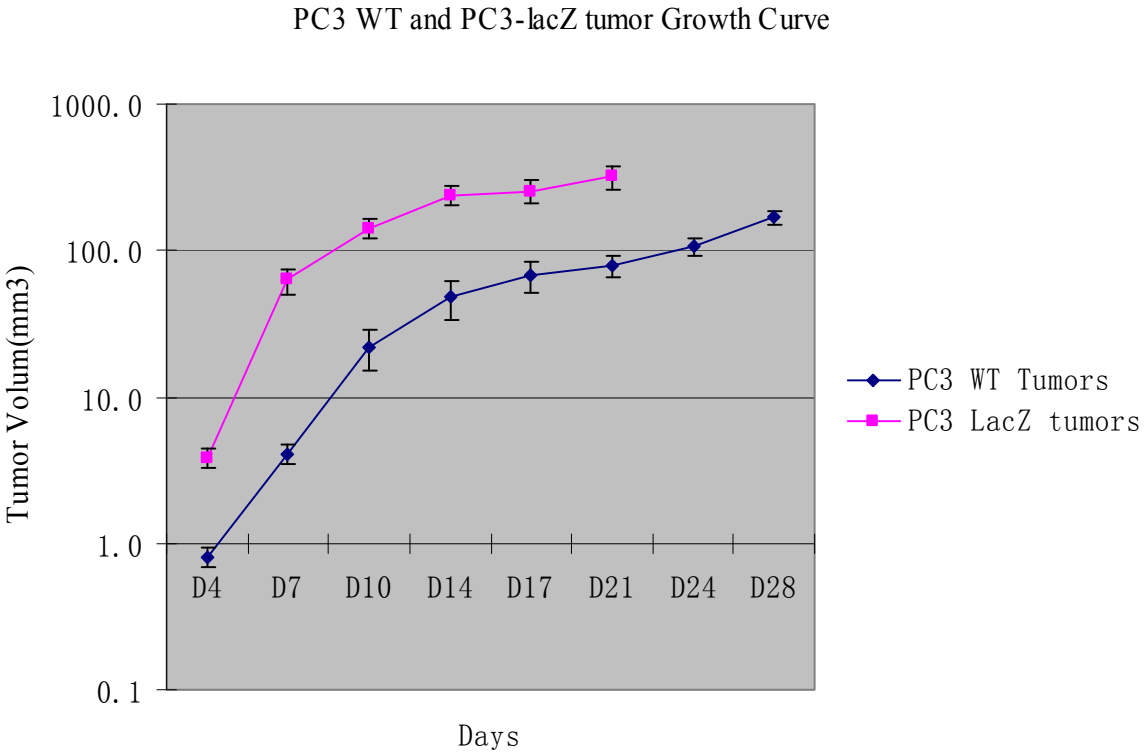


Fig6. Growth Curve of PC3 and PC3-lacZ tumor in Nude mice (n=6)

Task 5.Test synthetic phenyl galactoside substrates for β-galactosidase activity in transfected CaP cells (1X10^6) （Completed Year 2）

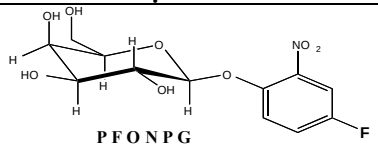
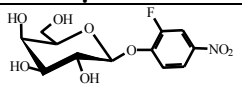
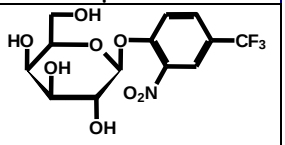
PC3-lacZ	**20.2μM/min**	**14.7μM/min**	31.5μM/min
Chemical structure	 PFONPG		 PC 3ONP

Fig.7 Hydrolytic rates of OFPNPG, PFONPG, PCF3ONPG with PC3-lacZ at 37°C in PBS.

I assessed several different synthetic phenyl galactoside substrates for β-galactosidase activity in PC3-lacZ cells and found PCF3ONPG has the highest hydrolytic rate.

Task 6. Perform feasibility experiments to assess the bystander effect of the mixture of transfected and non-transfected CaP cells *in vitro* (Completed Year 2)

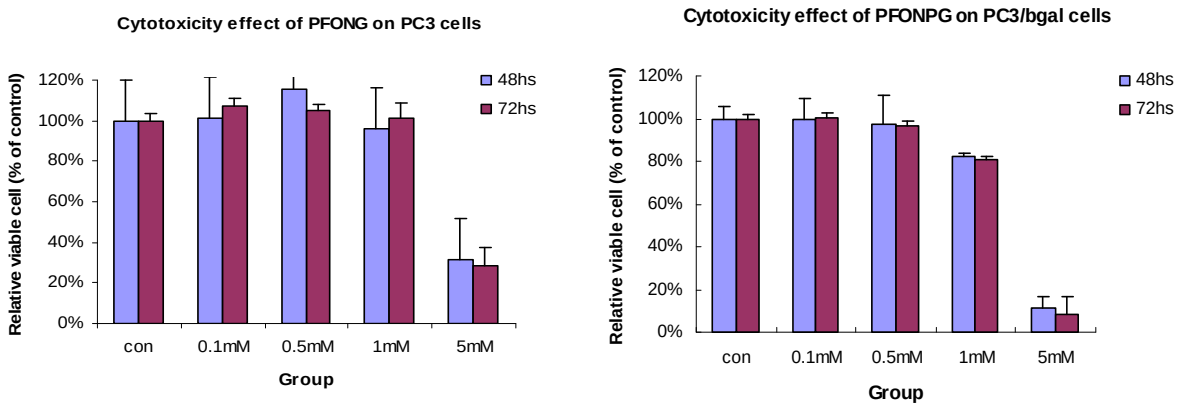


Fig8. Cytotoxicity of PFONPG on PC3 WT and PC3-lacZ cells

To further investigate the effect of PFONPG on PC3 and PC3-lacZ cell growth inhibition, $2x10^4$ cell suspensions was seeded in 24 well plate. After 24 hours, various concentrations of PFONPG were added. Cells were harvested of 48hrs and 72hrs and crystal violet methods were used to test the cell viability. Results show significant difference in 5mM between PC3 WT and PC3-lacZ cells. The aglycone PFONP, a close analog of the classic uncoupler DNP (dinitrophenol), is potentially cytolytic. The observation of enzyme activated cytotoxicity for PFONPG in PC3-lacZ tranfected cells at high concentration about 5mM. I tested several cells in both PC3 WT and lacZ expressing cells. They appeared to be slight difference. Therefore, I did not test mixed cell specific for the bystander effects.

Task 7. Examine the growth characteristic in each cell type implanted S.C *in vivo* (Completed Year 3)

I generated two new PC3-lacZ-luciferase and PC3-lacZ-GFP cell lines, which allowed correlative studies by bioluminescent imaging of tumor location and extent (Figure 9). An imaging approach based on a single reporter gene would be more convenient. I also tested Galacto-Light Plus^{TM} (Tropix) and beta-glo (Promega) to detect lacZ express in vitro and in vivo. In vitro deglycosylation releases a reactive oxitene which spontaneously decomposes emitting light. As expected, in culture WT cells gave no detectable light emission, but intense signal was detectable in β-gal expressing cells. In vivo, strong signal was detectable from lacZ tumors. This was further enhanced when a lysis buffer was included in the injection. For GFP and Luc, they

could be used to monitor tumor growth by optical imaging; lacZ could be used for gene therapy in the transfected double gene system.

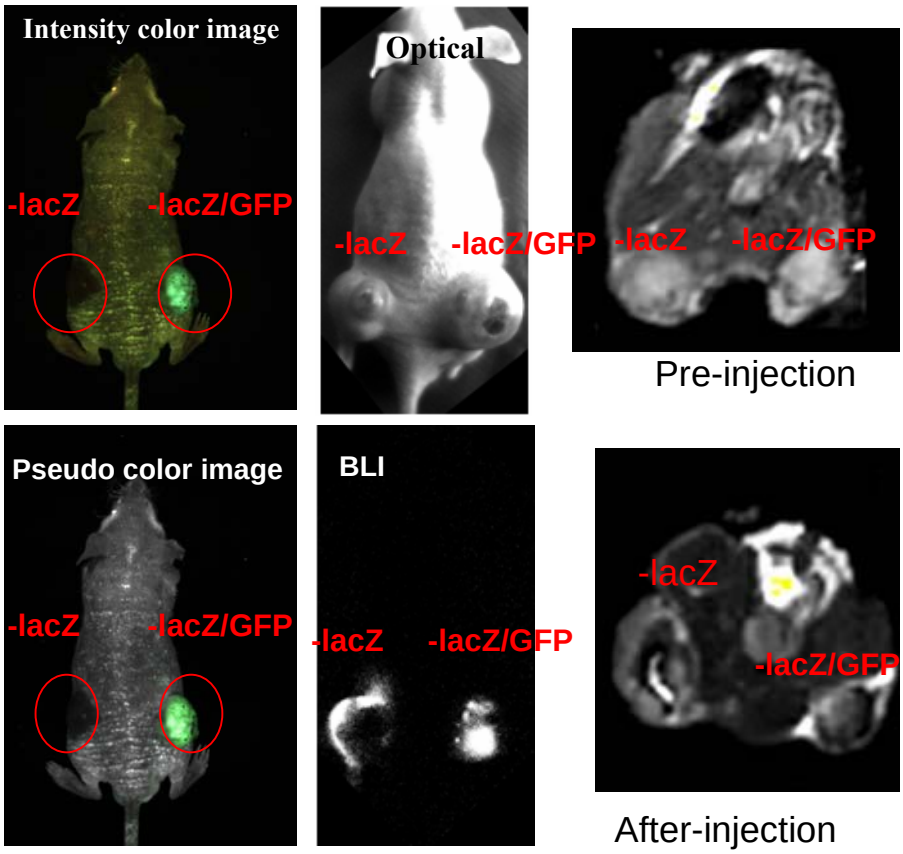


Fig. 9a. PC3 WT and PC3-lacZ tumors fluresent imaging (left) and BLI using Beta-glo (Middle) and ^{1}H MRI using S-gal +Ferric Ammonium Citrate (FAC) (Right)

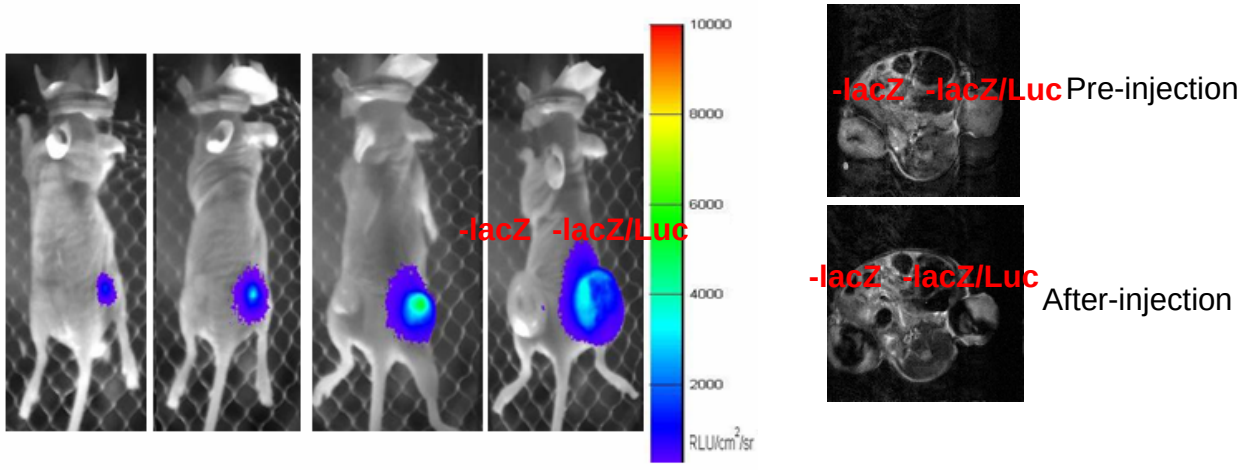


Fig. 9b. BLI of PC3-lacZ and PC3-lacZ-luc tumors (left) using D-luceand ^{1}H MRI using S-gal + Ferric Ammonium Citrate (FAC) (Right)

Task 8. Examine synthetic phenyl galactoside substrates for β-galactosidase activity and growth delay in implanted tumor *in vivo* (Completed Year 3)

1. PFONPG treatment for PC3 and PC3-lacZ tumor xenograft

(1)Low dose treatment: 40mg/kg PFONPG injected into two different group of mice twice weekly and continued treatment for 3 weeks. Measure tumors grow.

(2) High dose treatment: 200mg/kg PFONPG injected into two different groups' mice by IV twice weeks and continue treatment for 3 weeks, measure tumor grow.

(3) Saline as control: 100ul saline IV injected into control group. Same method is as treatment group mice. Every group n=6-8

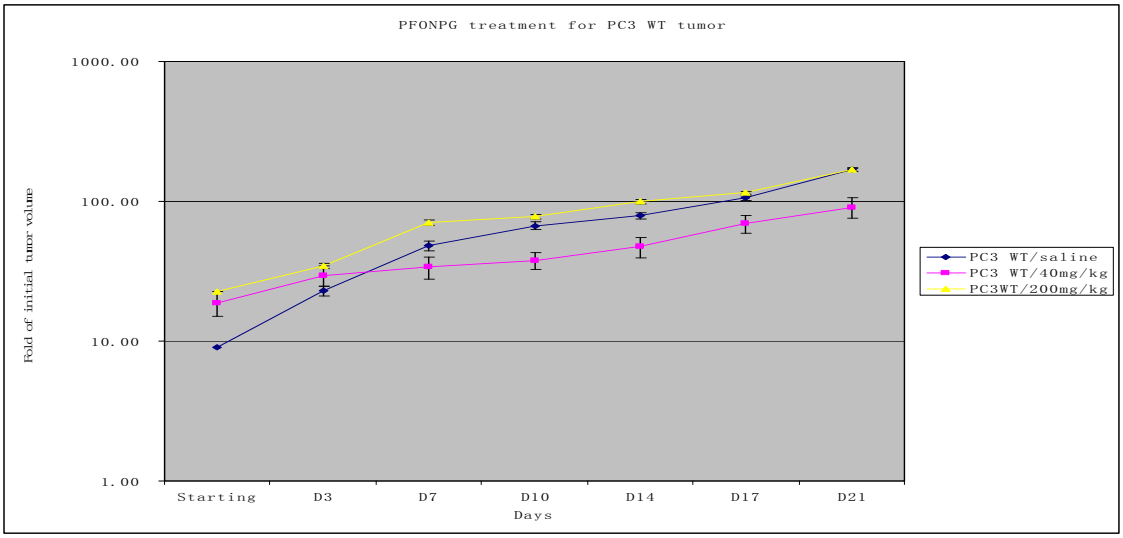


Fig10. PFONPG treatment for PC3 WT tumors group

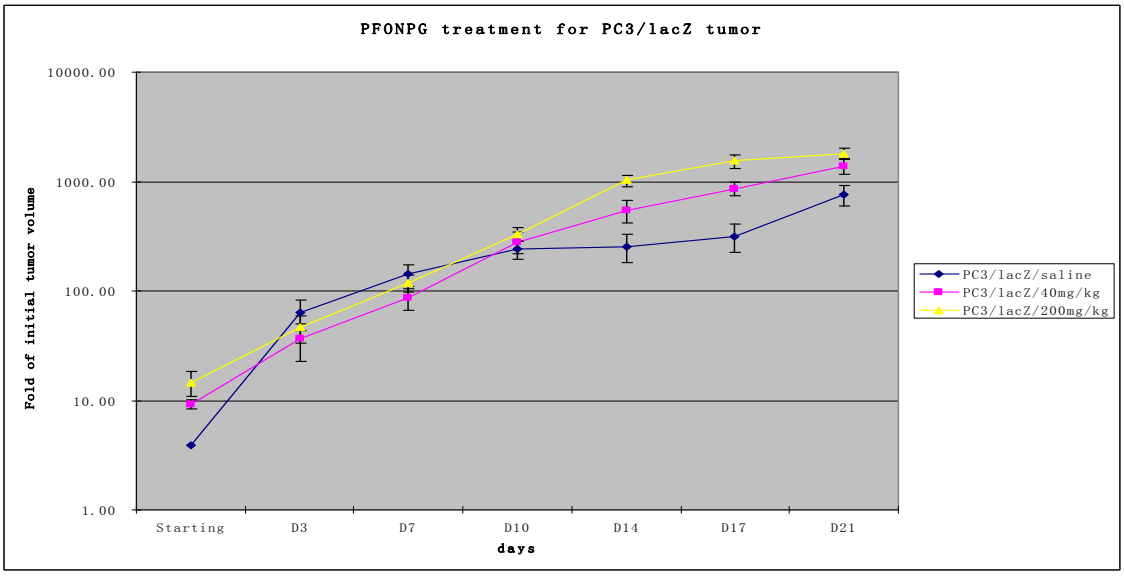


Fig.11 PFONPG treatment for PC3-lacZ tumors group

Comparing PFONPG treatment of the PC3 wild type to PC3-lacZ tumor groups, either a control or defined concentrations of PFONPG appeared nontoxic. It appeared that the transfected clone grew significantly faster than WT. We tried to synthesize a conjugate of 5FU with galactose, although parts of the synthesis were successful, inseparable mixtures resulted and enzyme activated toxicity in cell culture was not clear. However, we recently found that a conjugate of 5-Fluorouridine with galactose is commercially available and this was tested on PC3-lacZ and –WT cells (Figure 12).

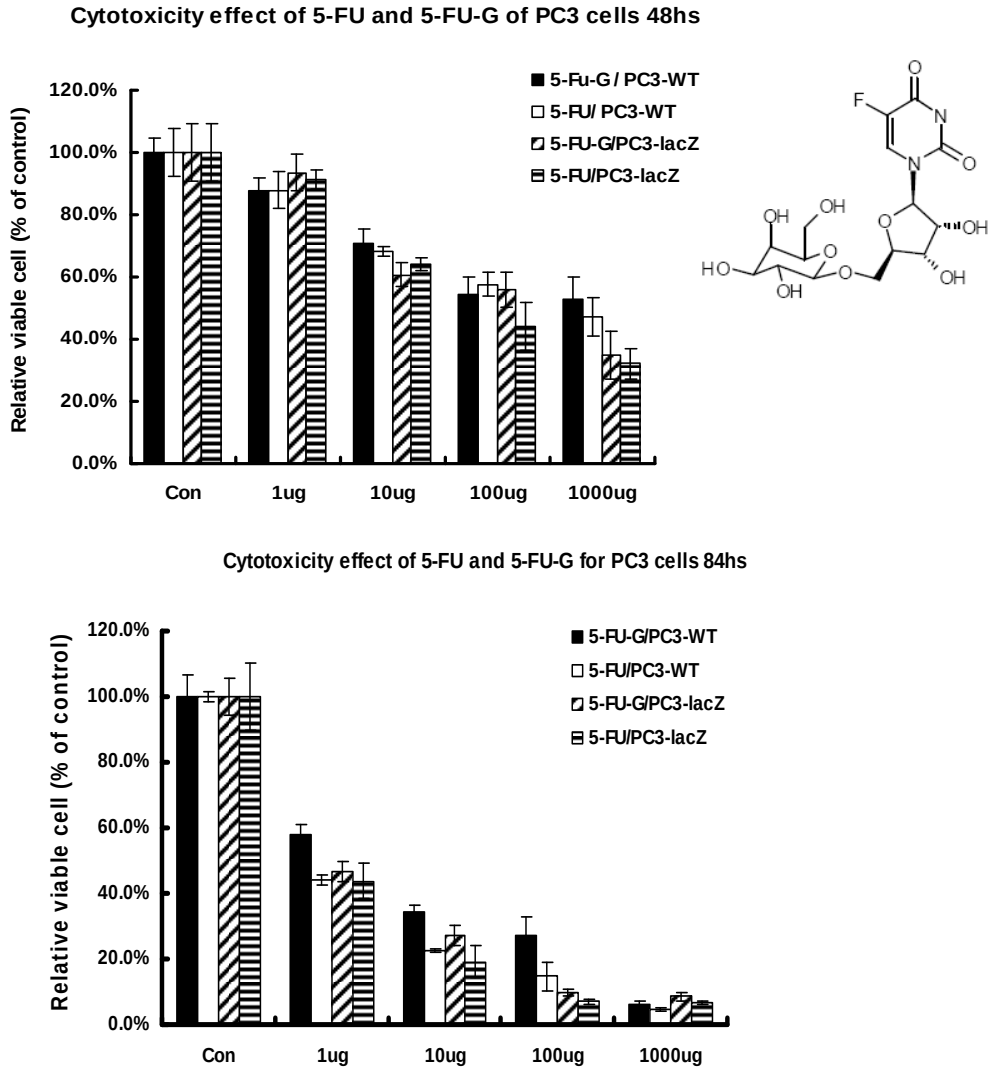


Fig 12. Cytotoxicity effect of 5-FU and 5-FU-G to PC3 and PC3-lacZ cells at 48hs and 84hs

5FU has a notoriously narrow window of efficacy. High concentrations cause systemic toxicity. We tested whether lacZ/β-gal could be used to activate and release 5FU in prostate tumors. Synthesis of pro-drugs was not successful in our lab, however, we recently became aware of and tested a commercial source of 5-Fluorouridine-5'-O-b-D-Galactopyranoside (5-FU-G). Intriguingly the lacZ expressing cells were more susceptible to both 5FU and the pro-drug. This may be a result of clonal selection during the transfection process. As expected, both cells types were more susceptible to 5FU than the pro-drug, longer time expose more toxic.

2. Novel Agent for ^{19}FNMR

To develop enhanced reporter molecules, diverse substrates were synthesized and the activity of β-gal is shown for 3 representatives. 2-Fluoro-4-nitrophenol-β-D-galactopyranoside (OFPNPG) belongs to a novel class of NMR active molecules (fluorophenyl-β-*D*-galactopyranosides), which are highly responsive to the action of β-gal. OFPNPG has a single ^{19}F peak at 55 ppm relative to aqueous sodium trifluoroacetate (NaTFA). Upon cleavage by β-gal, the pH sensitive aglycone OFPNP is observed at a chemical shift of 59-61 ppm.

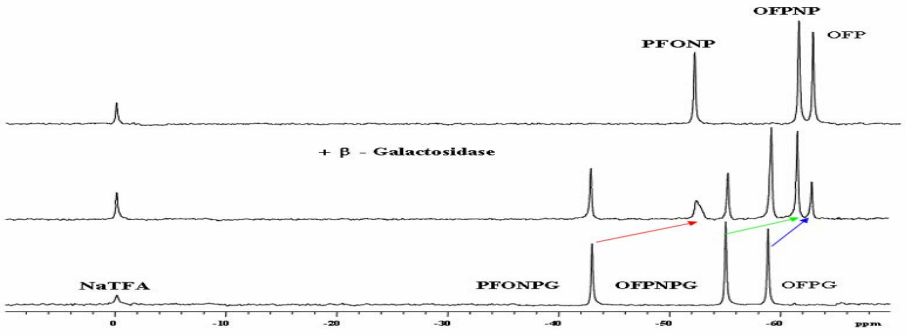


Fig.13. ^{19}F NMR diverse substrates for β-gal

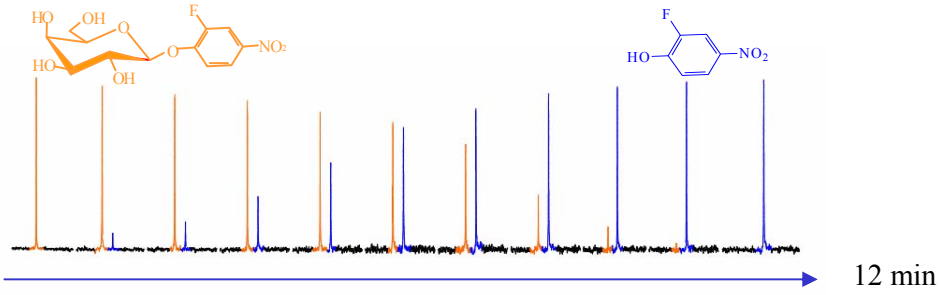


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Fig.14. Hydrolytic kinetics of OFPNPG by PC3-lacZ prostate cancer cells

5×10^5 PC3-lacZ cell in PBS buffer at 37°C (orange lines--signals of OFPNPG; blue lines-signals of product aglycone OFPNP). in PBS buffer at 37 °C

Over 12 min, substrate OFPNPG was converted to OFONP by β-gal enzyme activity in $5x10^5$ PC3-lacZ cell. Each spectrum was acquired in 1 min.

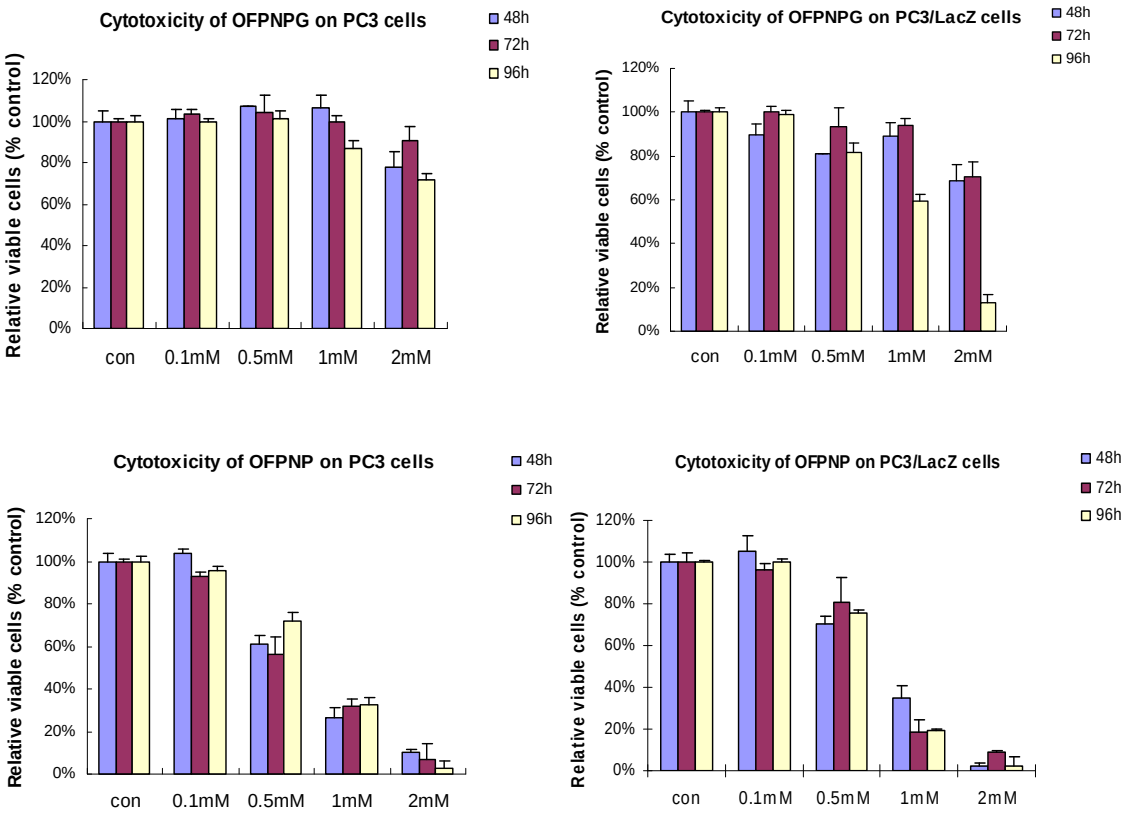

Fig.15. OFPNPG Cytotoxicity in PC3 WT and PC3-lacZ cells

PC3 WT and PC3-lacZ cell's viability for OFPNPG and OFPNP were detected by crystal violet methods; it is less toxic in PC3 WT cells than in PC3-lacZ cells for OFPNPG. The graphs show that only when the concentration of OFPNPG is at about 2mM, the PC3-lacZ cell had toxicity. But for the substrate OFPNP, both PC3 WT and PC3-lacZ are very toxic at 1 mM, only about 20% to 30% cells could be survived.

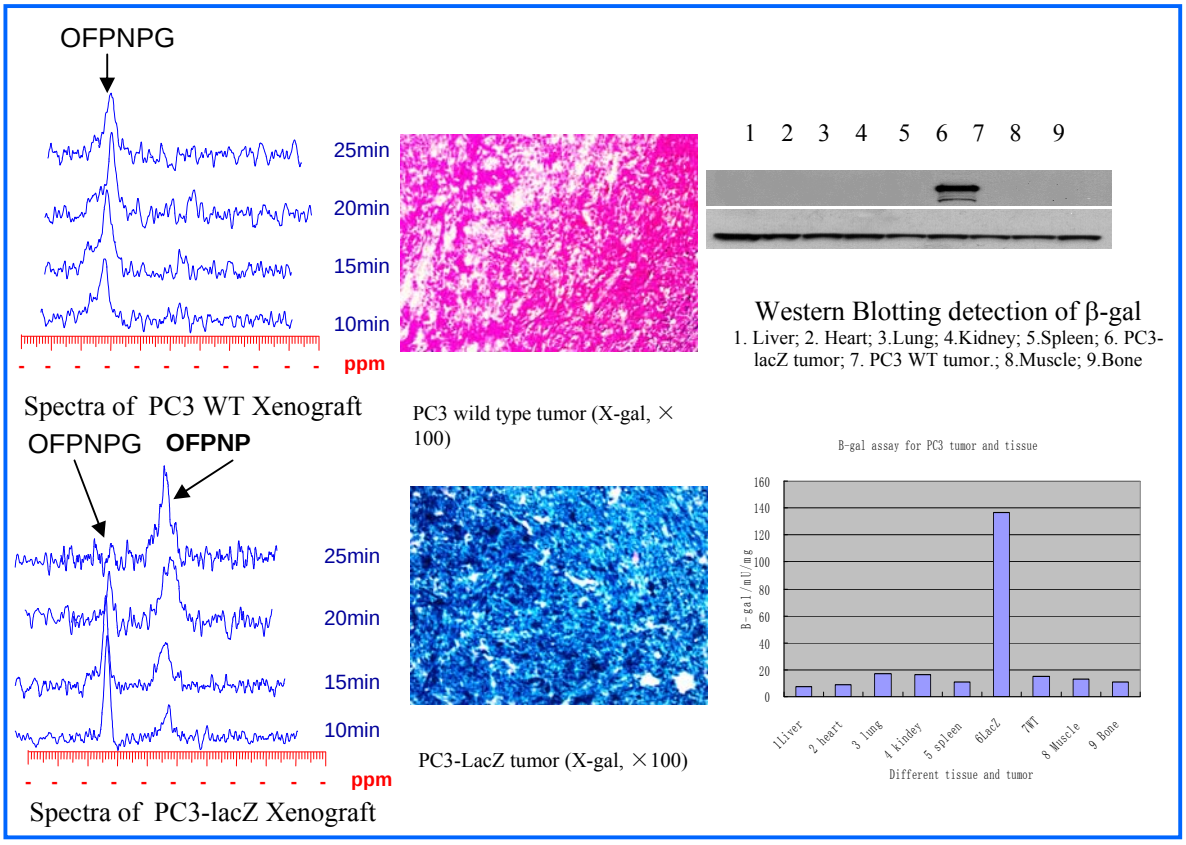

Fig 16. In vivo detection of lacZ gene expression in PC3 WT and PC3-lacZ tumors

PC3 wild type and PC3-lacZ cells were implanted subcutaneously in flanks of nude mice to grow as solid tumors. When the tumors reached about 200 mm^3 mice were anesthetized with isoflurane and OFPNPG (4 mg in 50 µl aqueous DMSO), containing sodium trifluoroacetate (Na-TFA, 10 mg/ml) as a standard, was injected intra tumorally (i.t.) using a fine 32G Hamilton syringe. ^{19}F spectroscopy was performed within 5 mins using a 4.7 T Varian scanner (188.2 MHz). Spectra were obtained with TR=1 s, na=128, SW=100 ppm and typically 30 Hz exponential line broadening was applied. Time course conversion of OFPNPG to OFPNP in PC3 and PC3-lacZ tumor and ^{19}F NMR signal of OFPNPG was readily detected with a signal to noise ratio >10 in 5 mins following direct intra-tumoral injection. Over a period of 30 min conversion of OFPNPG to product OFPNP was revealed by the development of a new up field signal unequivocally demonstrating β-gal activity. Rapid conversion of OFPNPG to OFPNP was seen in PC3- lacZ tumors, while little or none was seen in WT tumors. These results provide further evidence for the utility of this class of substrate to generate Reporter Products.

After ^{19}F NMR Spectroscopy of PC3 WT and PC3-lacZ Xenograft, the tumors were excised and cut up. One part was for histology and the other was for protein β-gal assay and Western blot. Comparing histology, β-gal assay and Western blot results, I found PC3 WT has low expression, PC3-lacZ has high expression for lacZ gene. I found minimal β-gal expression in other organs. These results provide our first observations that the chemical shift response is sufficient to observe gal activity by ^{19}F NMR in PC3 human prostate tumor xenografts in mice. This approach directly reveals β-gal activity, which could be used in tandem with therapeutic genes to monitor therapy. As gene therapy becomes a reality, the ability to detect transgene expression non-invasively will become increasingly important for treatment planning and optimization. OFPNPG is a promising lacZ gene reporter molecule for ^{19}F MR spectroscopy. Meanwhile, we are also developing new generations of ^{19}F NMR reporter designed to minimize toxicity.

Task 9. Prepare manuscripts and reports (Completed year 3)

Final report provided and manuscripts and conference abstracts follow.

KEY RESEARCH ACCOMPLISHMENTS:

- I constructed different mammary cells expression lacZ recombinant vectors
- I generated prostate tumor cell lines stably expressing high activity of β-galactosidase.
- I developed the double gene PC3-lacZ-luc prostate cancer cell lines, and detect lacZ and luc double gene expression by BLI and ^{1}H MRI
- I developed the other double gene PC3-lacZ-GFP prostate cancer cell lines, and detected lacZ gene by BLI (beta-glo based) and ^{1}H MRI (S-gal +FAC)
- I successfully used direct intra tumoral injection of ^{19}F NMR substrates allows detection of lacZ expressing prostate tumors versus wild type.
- Career developed: I gained the skills to be included in a new NCI R21 MAGIC grant etc, which seeks to develop novel β-gal reporter molecular.

Reportable Outcomes

Publications:

1. **L. Liu**, V. Kodibagkar, J.Yu, and R.P. Mason, ^{19}F-NMR detection of lacZ gene expression via the enzymic hydrolysis of 2-fluoro-4-nitrophenyl β-galactopyranoside in vivo in PC3 prostate tumor xenografts in the mouse, , *Faseb J*., 2007, 21, on line March 9
2. V. Kodibagkar, J.Yu, **L. Liu** and R.P. Mason, Imaging β-galactosidase activity using 19F chemical shift imaging of LacZ gene-reporter molecule 2-fluoro-4-nitrophenol-β-d-galactopyranoside, *Magn. Reson. Imaging,* 2006, 24, 959–962
3. J. Yu, **L. Liu**, V. Kodibagkar, W. Cui and R. P. Mason, Synthesis and Evaluation of Novel Enhanced Gene Reporter Molecules: Detection of β-Galactosidase Activity Using ^{19}F NMR of Trifluoromethylated Aryl β-D-Galactopyranosides, *Bioorg Med Chem*. 2006,14(2):326-333

Conference abstracts:

1. **L. Liu**, J. Yu, V. Kodibagkar, S. L.Brown and R. P. Mason, Using 2-fluoro-4-nitrophenyl beta-gal-D-galactopyranoside to detect beta-galactosidase in PC3 Prostate Xenograft by ^{19}F NMR, DOD prostate cancer meeting, Atlanta, Sep. 2007
2. **L. Liu**, V. Kodibagkar, J. Yu and R. P. Mason, In vivo detection of β-Galactosidase expression in PC3 prostate xenograft tumor by ^{19}F NMR, SMI 5th meeting, Hawaii, Aug, 2006, # 372
3. **L, Liu**, V. Kodibagkar, J. Yu and R. P. Mason, In vivo detection of *lacZ* gene expression in PC3 prostate xenograft tumor by ^{19}F NMR, AACR 97th meeting, Washington DC, April, 2006
4. W, Cui, **L, Liu**, P. Peschke, U. Haberkorn, R. P. Mason, Transmembrane pH gradients in tumor cells: observations using ^{19}F NMR of promising new reporter molecules, ISMRM 14th *Scientific Meeting in* Seattle, Washington,May,2006
5. R. P. Mason, J. Yu, **L. Liu**, W. Cui and V. Kodibagkar, Novel Magnetic resonance Assays of Gene Imaging Constructs (MAGIC), Imaging in 2020, Jackson Hole, Wyoming, September 2005.
6. J. Yu, **L. Liu,** V. D. Kodibagkar, W. Cui, R. D. Gerard, R. P. Mason, Novel "Smart" ^{1}H MRI Contrast Agents for Assessing *lacZ* Gene Expression" *ISMRM 13th Scientific Meeting in Miami Beach*, Florida, USA May 2005.
7. W. Cui, **L. Liu**, J. Yu, R. P. Mason, Detection of β-Galactosidase Activity in a Human Tumor Xenograft by ^{1}H MRI in vivo Using S-Gal^{TM} , *ISMRM 13th Scientific Meeting in Miami Beach*, Florida, USA May 2005.

8. V. Kodibagkar, J. Yu, **L. Liu**, S. Brown, H. P. Hetherington, R. D. Gerard, and R. P. Mason, ^{19}F CSI of gene-reporter molecule OFPNPG", I*SMRM 13th Scientific Meeting in Miami Beach*, Florida, USA May 2005.
9. **L. Liu,** V. Kodibagkar, J. Yu and R. P. Mason, **In vivo** detection of lacZ gene expression in a human prostate xenograft tumor by ^{19}F NMR CSI using OFPNPG, *Molecular Medicine Symposium*, Houston, Feb. 2005

Conclusions

1. I developed a series of recombinant lacZ gene expression vectors
2. I screened several stable expression of lacZ cell lines, MAT-Lu-*lacZ* and PC3-*lacZ* cells
3. I developed two double gene systems, PC3-lacZ-GFP and PCe-lacZ-Luc
4. I confirmed high expression of *lacZ* gene in these cells by beta-gal activity assay X-gal staining and West blotting.
5. I obtained the growth curve PC3 and PC3-*lacZ in vitro* and *in vivo.*
6. PFONPG treatment for PC3 and PC3-lacZ tumor
7. Developed novel ^{19}F NMR lacZ gene reporter molecules.
8. I evaluated selective gene activated cytotoxicity.
9. I have also learnt techniques related to tumor implantation, treatment, small animal handling and MRI.

Appendices

The FASEB Journal article fj.06-7366lsf. Published online March 9, 2007.

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^{19}F-NMR detection of *lacZ* gene expression *via* the enzymic hydrolysis of 2-fluoro-4-nitrophenyl β-D-galactopyranoside *in vivo* in PC3 prostate tumor xenografts in the mouse[1]

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ABSTRACT **Gene therapy shows promise for treating prostate cancer and has been evaluated in several clinical trials. A major challenge that remains is to establish a method for verifying transgene activity *in situ*. The *lacZ* gene encoding β-galactosidase historically has been the most popular reporter gene for molecular biology. We have designed a ^{19}F NMR approach to reveal *lacZ* gene expression by assessing β-galactosidase (β-gal) activity *in vivo*. The substrate 2-fluoro-4-nitrophenyl β-D-galactopyranoside (OFPNPG) is readily hydrolyzed by β-gal with a corresponding decrease in the ^{19}F-NMR signal from OFPNPG and the appearance of a new signal shifted 4–6 ppm upfield from the aglycone 2-fluoro-4-nitrophenol (OFPNP). We report proof of principle in cultures of PC3 prostate cancer cells using ^{19}F NMR spectroscopy and ^{19}F chemical shift imaging. More importantly, we demonstrate for the first time the ability to differentiate wild-type and *lacZ*-expressing prostate tumor xenografts in mice using this approach.—Liu, L., Kodibagkar, V. D., Yu, J-X., Mason, R. P. ^{19}F-NMR detection of *lacZ* gene expression *via* the enzymic hydrolysis of 2-fluoro-4-nitrophenyl β-D-galactopyranoside *in vivo* in PC3 prostate tumor. *FASEB J.* 21, 000–000 (2007)**

Key Words: β-galactosidase · ^{19}F CSI · ^{19}F MRS · gene reporter

GENE THERAPY SHOWS PROMISE for treating cancer and has been tested in several clinical trials for the prostate (1–8). A major hurdle is to establish a method of verifying transgene activity *in situ*; various reporter genes have been developed (9, 10), in some cases using a single gene as both therapeutic and reporter (*e.g.*, thymidine kinase or cytosine deaminase) (11–13). The *lacZ* gene encoding the enzyme β-galactosidase (β-gal), first described by Jacob and Monod (14), remains a popular reporter gene in molecular biology. PCR and Western blot are the most commonly used techniques for evaluation of gene expression, and can be used for quantitation, but are highly invasive (requiring a biopsy). Multiple colorimetric reporter substrates for β-gal have been demonstrated, and some are commercially available for histology and *in vitro* detection (15–19).

However, current methods of detecting β-gal activity are generally not suitable for applications *in vivo*. Therefore, development of reporter molecules for noninvasive *in vivo* detection of *lacZ* transgene expression would be of considerable value both for research and future clinical gene therapy trials. A characteristic of β-gal is its extreme promiscuity (lack of substrate specificity), which can be exploited with a variety of substrate structures. Recently, Tung *et al.* (20) presented an optical near-infrared fluorescence approach based on 9*H*-(1,3-dichloro-9,9-dimethylacridin-2-one-7-yl) β-D-galactopyranoside and detected β-gal expression in xenografts in mice. Lee *et al.* (21) described a radiolabeled substrate 2-(4-[^{125}I/^{123}I]iodophenyl)ethyl-1-thio-β-D-galactopyranoside, which was used to detect β-gal activity in mice using a gamma camera. Louie *et al.* (22) reported a Gd(III)-based ^{1}H MRI approach using 1-[2-(β-D-galactopyranosyloxy)propyl]-4,7,10-tris(carboxymethyl)-1,4,7,10-tetraazacyclododecane) gadolinium (III) to assess β-gal activity in developing tadpoles.

We previously presented the successful synthesis and evaluation of fluoro-nitro-phenyl-galactopyranosides to detect β-gal activity *in vitro* by ^{19}F NMR spectroscopy and imaging (23–28). In particular, 2-fluoro-4-nitrophenyl β-D-galactopyranoside (OFPNPG) is highly responsive to the action of β-gal, and cleavage to form the aglycone 2-fluoro-4-nitrophenol (OFPNP) results in a pH-dependent chemical shift of 4–6 ppm for the fluorine resonance. Our previous investigations focused on breast cancer or transiently transfected prostate cancer cells. We now report application to stably transfected human PC3 prostate tumor cells; most importantly, we demonstrate *in vivo* application of

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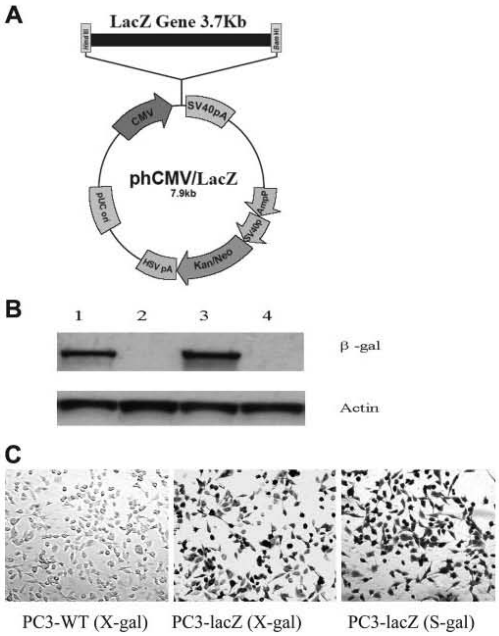

Figure 1. Generation of PC3 cells stably expressing of β-gal. *A)* Map of recombinant *lacZ* vector (phCMV/*lacZ*), *B)* Western blot: cell extracts of two transfected lines PC3-*lacZ*1 (lane 1) and PC3-*lacZ* (lane 3), together with PC3-WT (lanes 2 and 4), were examined. *C)* PC3 wild-type and PC3-*lacZ* cells were stained using X-gal and S-gal: >90% of PC3-*lacZ* cells were stained blue or black, respectively, whereas the PC3 wild type cells did not stain.

PC3-*lacZ* clone showed the highest *lacZ* expression and was used for all further investigations. When stably transfected PC3-*lacZ* cells and wild-type PC3 counterparts were stained using X-gal or S-gal, >90% of PC3-*lacZ* cells stained blue or black, respectively, after 30 passages in culture; the PC3 wild-type cells did not stain (Fig. 1*C*).

OFPNPG has a single sharp resonance at −54.93 ppm with respect to dilute sodium trifluoroacetate. The spin-lattice relaxation time T1 = 0.74 ± 0.03 s at 9.4 T. Cleavage by β-gal releases the OFPNP, which has a pH-sensitive chemical shift of −61 ppm at pH 7.4 (range $\delta_{(acid)}$ −58.77 ppm, $\delta_{(base)}$ = −61.01 and pKa=6.03) and a spin-lattice relaxation time T1 = 2.33 ± 0.04 s, which is considerably longer than for the substrate.

Addition of OFPNPG to a suspension of PC3-*lacZ* cells at 37°C led to rapid cleavage of the galactoside and release of the yellow aglycone OFPNP. Both substrate and product were detectable by ^{19}F-MRS, and a time course is shown in **Fig. 2**. Loss of substrate signal is accompanied by the appearance of the algycone product 4–6 ppm upfield. The ultimate intensity of the product is less than that of the substrate due to partial spectral saturation, since T1 of the algycone is about three times longer. Since OFPNP could act as a toxic ionophore, both the substrate and aglycone were tested for toxicity. At 0.5 mM, OFPNP showed considerable toxicity toward both lacZ and WT cells, with a 60 to 80% survival after 96 h exposure and only 5% survival for 2 mM. The substrate was much less toxic with essentially no toxicity after 96 h for 0.5 mM. At 2 mM there was an ~80% survival after 96 h for WT cells, whereas considerable cell loss was observed after 96 h exposure to concentrations of >1 mM for the *lacZ* cells with only 10% survival at 2 mM. Conversion of OFPNPG to OFPNP by stably transfected human prostate cancer PC3-*lacZ* cells could also be detected by ^{19}F CSI (**Fig. 3**). The addition of 10^7 PC3-*lacZ* cells to a 70 mM solution of OFPNPG resulted in ~40% conversion to OFPNP after 4 h.

Direct injection of OFPNPG (3.9 mg in 50 μl aqueous DMSO) into a tumor (~1.7 cm^3, providing a concentration of ~6.5 mM) yielded a ^{19}F-MRS signal, which was readily detected with a signal-to-noise ratio of ~20–30 in 4 min; OFPNPG and OFPNP signals were easily distinguishable at 4.7 T (**Fig. 4**). Over a period of 30 min, the PC3-*lacZ* tumor converted ~80% of OFPNPG to OFPNP demonstrating β-gal activity (Fig. 4*A*). No visible OFPNP signal was detected over the same period in PC3-WT tumor (Fig. 4*B*). After MRS, histology and protein analysis were performed on excised tissue. Histological sections from PC3-*lacZ* showed >90% of tissue stained blue with X-gal for β-gal (Fig. 4*C*), whereas PC3-WT tumor showed little or no blue stain (Fig. 4*D*). The β-gal assay and Western blot were performed on tissue from liver, heart, lung, kidney, spleen, PC3-*lacZ* tumor, wild-type tumor, muscle, and bone. High β-gal activity was detected in PC3-*lacZ* tumor alone with little activity in wild-type tumor or normal tissues (Fig. 4*E, F*).

DISCUSSION

We previously demonstrated that fluorophenyl β-D-galactopyranosides could be used to detect β-gal activity

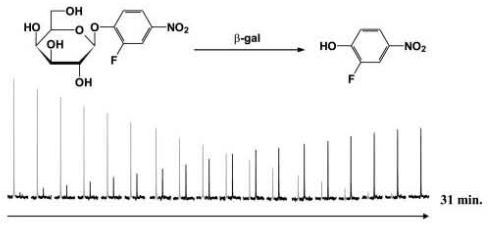

Figure 2. Conversion of OFPNPG to OFPNP by stably transfected PC3-*lacZ* cells. Stacked plot series showing the conversion of OFPNPG (2.9 mg; 9 mmol) by PC3-*lacZ* prostate cancer cells (2×10^7 in PBS, pH=7.4 at 37°C) to the aglycone OFPNP, which resonates ~5 ppm upfield of the substrate (OFPNPG, gray traces, OFPNP black traces). Sequential spectra were acquired in 102 *s* each over a period of 31 min.

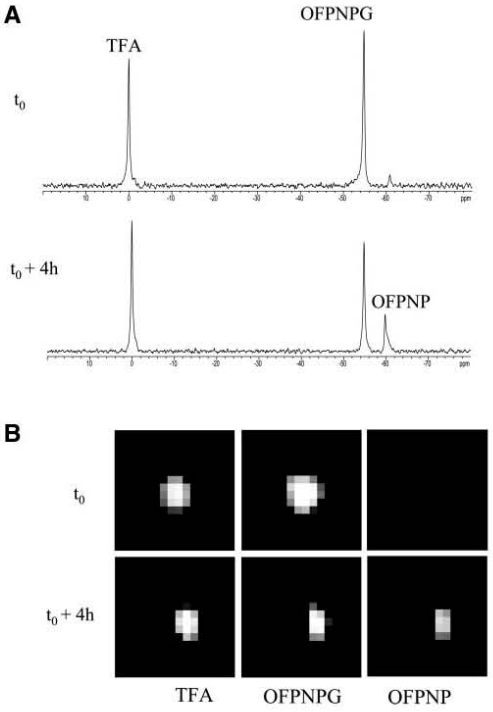

Figure 3. Conversion of OFPNPG to OFPNP by PC3-*lacZ* cells detected using ^{19}F-CSI at 4.7 T. PC3-*lacZ* cells (10^7) were added to a 1 cm diameter vial containing OFPNPG (15.6 mg; 48 mmol (70 mM) and imaged over a period of 4 h at the ambient temperature of the magnet bore (~16°C): field of view = 30 × 30 mm, spectral width = 70 ppm, one slice with thickness = 10 mm, data matrix = 16 × 16 voxels, TR/TE = 1000/12 ms, 4 averages. *A)* Bulk spectra and *B)* chemical shift images each acquired in 5 min.

by ^{19}F-MRS of cells and identified OFPNPG as the best candidate among several simple analogs (24). Upon cleavage by β-gal, the substrate OFPNPG releases the aglycone product OFPNP, which has a pH-dependent ^{19}F chemical shift of $\Delta\delta = -4$ to -6 ppm relative to the substrate (24). The pKa of the aglycone is 6.0 and the $\Delta\delta$ at pH 7 is ~ -5.0 ppm, so that the signal is well separated from that of the substrate, allowing spectroscopic resolution and chemical shift imaging of each species at 4.7 T with no overlap between the two peaks. Our new results indicate that the chemical shift difference is sufficient to observe β-gal activity by ^{19}F CSI in stably transfected PC3-*lacZ* cells, though currently the signal-to-noise obtained at 4.7 T with a 2 cm resonator does not permit *in vivo* imaging.

The ^{19}F-MRS approach is shown to be feasible *in vivo* for prostate tumor xenografts with a volume of > 1 ml growing in the mouse. Here, we achieved an SNR in the range 20–30 in 4 min based on an injection of 3.9 mg of substrate. Assuming uniform distribution of reporter throughout the tumor this represents a concentration of ~6.5 mM, which is expected to be cytotoxic. However, one could expect to achieve an SNR of ~5–8 with 0.5 mg substrate (<1 mM) in 16 min causing little or no toxicity. Further improvement in SNR is possible with the use of size-matched surface coils. The product aglycone line width is broader than the substrate *in vivo*. This may be attributed to the pH dependency of the chemical shift and would be influenced by the expected extra- plus intracellular distribution and pH heterogeneity of the tumor. Ultimately, substrate and product would be expected to wash out of the tumors, but here the substrate intensity was found to be quite stable in wild-type tumor over a period of 25 min. Most important, the comparison between *lacZ* transfected and wild-type PC3 xenografts by ^{19}F-MRS of OFPNPG matches the histological results for β-gal staining.

^{19}F-MRS is becoming increasingly popular for *in vivo* investigations because the ^{19}F nucleus exhibits an exceptionally large, structure-dependent chemical shift range and because there is essentially no endogenous background signal (30). Here, we exploited OFPNPG with the fluorine substituent at the *ortho* position relative to the glycosidic linkage. However, we believe superior molecules can be developed. We have tested the analog with a CF_3 moiety in place of the single fluorine atom and found the expected enhanced signal-to-noise ratio. However, the chemical shift response to bond cleavage was much smaller ($\Delta\delta < 1.2$ ppm) (26). ^{19}F CSI was achieved by deconvolution for cultured cells, but the small $\Delta\delta$ may be a problem for *in vivo* imaging. We were able to reduce molecular toxicity by using a fluoropyridoxol aglycone reporter in place of the fluoronitrophenol, but the synthesis is more complex; the substrate has poor water solubility and the enzyme-catalyzed reaction was much slower (25). These issues could be addressed by polyglycosylation (27), and we are investigating such complex substrates.

A particular problem with the OFPNPG/OFPNP approach is the need for direct intratumoral injection. We are currently addressing this issue by developing agents that release trapped or precipitated aglycone products (*e.g.*, 2-[(β-D-galactopyranosyl) oxy]-3-fluorocatechol; ref. 31), with the goal of accumulating the diagnostic product by analogy with nuclear medicine techniques that detect trapped phosphorylated agents (10). While intratumoral injection is not ideal, we believe it represents progress for NMR reporter approaches to β-gal. The previous approach of Louie *et al.* (22) required direct intracellular injection, with the trapped molecule being used to follow cell lineage in tadpole development.

Since ^{19}F NMR has been used to monitor the conversion of 5-fluorocytosine to 5-fluorouracil (5-FU) in cells (11) and tumors (12), one might question the need for a reporter gene approach to *lacZ*. However, *lacZ* remains one of the most widely used reporter genes in molecular biology, and it may be useful to exploit a gene that does not generate a cytotoxic product (*viz.* 5-FU). Therefore, we continue

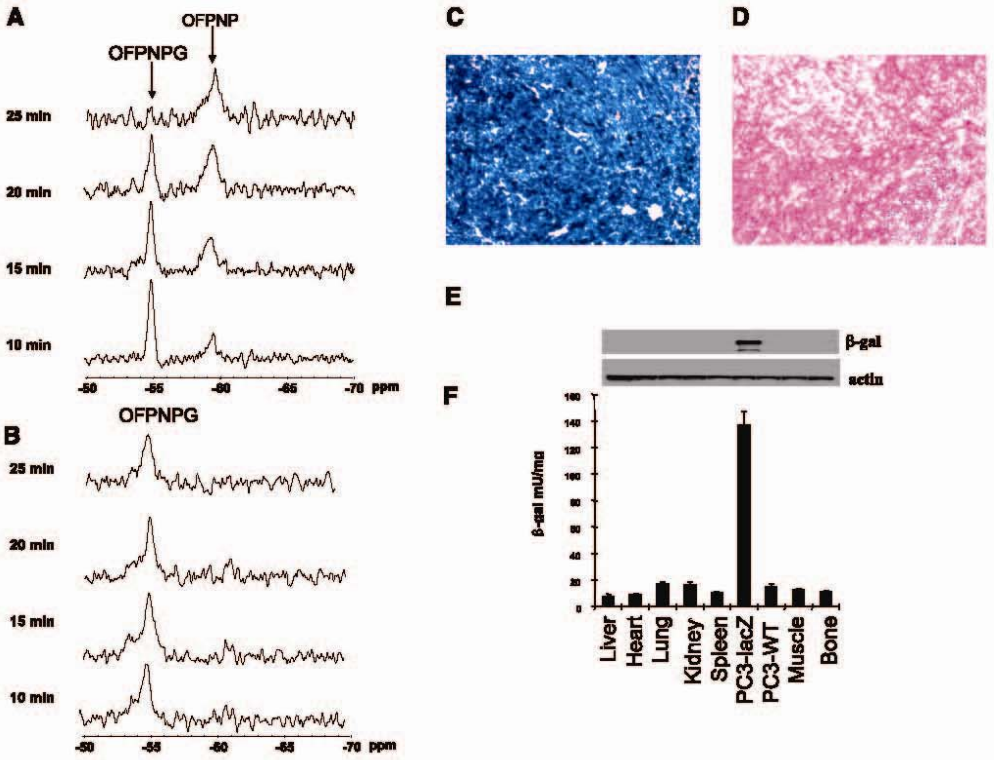

Figure 4. *In vivo* detection of β-gal expression. Time courses for ^{19}F-MRS spectra obtained from solid tumors *in vivo* (4.7 T) using a 2 cm single-turn volume coil, following direct intratumoral injection of OFPNPG (3.9 mg in 50 μl aqueous DMSO). *A)* PC3-*lacZ* tumor (dimensions 1.4 cm×1.5 cm×0.8 cm). *B)* PC3-WT tumor (dimension 1.3 cm×1 cm×0.6 cm) and X-gal staining for *C)* PC3-*lacZ* and *D)* PC3-WT tumor sections, respectively. Tumors and tissues (liver, heart, lung, kidney, spleen, PC3-*lacZ* tumor, PC3-WT tumor, muscle, and bone) were also examined using *E)* Western blot showing bands for β-gal and actin (as control) and *F)* β-gal assay.

to seek reporters with lower toxicity. As a corollary, our approach could stimulate new approaches for detecting gene-directed enzyme prodrug therapy or antibody-directed prodrug therapy (32, 33). Instead of seeking to minimize product toxicity, we may seek to generate a highly toxic product that is liberated locally by β-gal activity.

We believe that noninvasive *in vivo* detection of gene reporter molecules will become increasingly important in biomedicine and that it will be important to have diverse agents, genes, and modalities for specific applications. Fluorophenyl β-D-galactosides offer a novel approach for detecting β-gal activity. This study provides the first evidence for the utility of OFPNPG as a gene reporter molecule for investigations in animals. It demonstrates a novel approach and increases the diversity of tools for potential evaluation of gene therapy. FJ

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Using 2-fluoro-4-nitrophenyl beta-gal-D-galactopyranoside to detect beta-galactosidase in PC3 Prostate Xenograft by ^{19}F NMR

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Prostate cancer is one of the most common malignant tumors with increasing incidence in aging men and it presents a formidable public health problem. Gene therapy has been successfully exploited in several clinical trials. A major challenge is to establish a method of verifying transgene activity *in situ*. The lacZ gene, coding beta-galactosidase (beta-gal), has historically been the most popular reporter gene for molecular biology, and many colorimetric reporter substrates have been demonstrated: some are commercially available for histology and *in vitro* detection. We have now demonstrated proof of principle for detection of beta-gal activity in prostate tumors *in vivo* using ^{19}F NMR.

In order to undertake these investigations, we required stably transfected prostate cancer cells. PC3-lacZ tumor cells were generated by recombinant plasmid phCMV/lacZ transfection and a high expressing clone selected. Diverse substrates were synthesized as potential reporter molecules and 2-fluoro-4-nitrophenyl beta-D-galactopyranoside (OFPNPG) was identified as having high activity towards β-gal and minimal toxicity. This substrate belongs to a novel class of NMR active molecules (fluorophenyl beta-D-galactopyranosides). Of course, the aglycones are close analogs of the classic uncoupler DNP (dinitrophenol) prompting us to investigate whether there would be enzyme activated cytotoxicity. In cell culture phenylgalactopyranosides showed significantly higher toxicity towards beta-gal expressing cells than WT. However, investigating therapy in PC3-lacZ tumors *in vivo* in mice showed no significant different for PC3 and PC3-lacZ tumors.

As a reporter, OFPNPG is readily hydrolyzed by beta-gal with a corresponding loss of ^{19}F NMR signal and appearance of a new signal shifted 4-6 ppm upfield attributable to the liberated aglycone 2-fluoro-4-nitrophenol (OFPNP). When a solution of OFPNPG (4 mg in 50 μl aqueous DMSO) was injected intra-tumorally in PC3 wild type and PC3-lacZ tumors, ^{19}F NMR signal was readily detected at 4.7 T. Over a period of 30 min conversion of OFPNPG to product OFPNP was observed unequivocally demonstrating beta-gal activity. Tumor and tissues were

also examined by Western blots and beta-gal assay for activity. High β-gal activity was found in the PC3-lacZ tumor, with minimal activity in normal tissues.

IMPACT: This approach directly reveals beta-gal activity, which could be used in tandem with therapeutic genes to monitor therapy. As gene therapy becomes a reality, the ability to detect transgenic expression non-invasively will become increasingly important for treatment planning and optimization. We report proof of principle in cultures of PC3 prostate cancer cells using ^{19}F NMR spectroscopy and ^{19}F Chemical Shift Imaging (CSI). More significantly, we demonstrate for the first time the ability to differentiate wild-type and lacZ-expressing prostate tumor xenografts in mice using this approach.

In addition these studies laid a foundation enabling us to consider proton MRI approaches to detect both beta-gal and PSMA activity using novel reporter molecules.

Keywords: Beta-galactosidase, ^{19}F NMR, lacZ gene, prostate cancer, gene therapy
Supported by DOD PC031075 (LL)

Detection of *lacZ* Gene Expression in PC3 Prostate Xenograft by ^{19}F NMR

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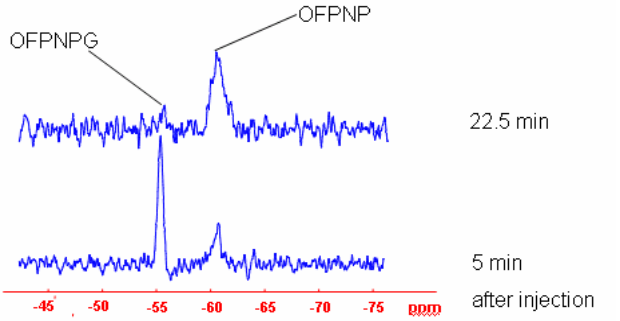
Gene therapy shows promise for treating prostate cancer and has been successfully exploited in several clinical trials. A major hurdle is establishing a method of verifying transgene activity *in situ*. β-galactosidase (β-gal) has historically been the most popular reporter gene for molecular biology. We are designing non-invasive NMR approaches to reveal β-gal activity *in vivo*. 2-Fluoro-4-nitrophenol-β-D-galactopyranoside (OFPNPG) belongs to a novel class of NMR active molecules (fluorophenyl-β-*D*-galactopyranosides), which are highly responsive to the action of β-gal. OFPNPG has a single ^{19}F peak at 55 ppm relative to aqueous sodium trifluoroacetate (NaTFA). Upon cleavage by β-gal, the pH sensitive aglycone OFPNP is observed at a chemical shift of 59-61 ppm. We now show the chemical shift response is sufficient to observe β-gal activity by NMR in PC3 human prostate tumor xenografts in mice.

PC3-lacZ tumor cells were generated by recombinant plasmid phCMV/lacZ transfection and a high expressing clone selected. Cells were implanted in the flank of nude mice and allowed to grow to about 1cm^3. When a solution of OFPNPG (4 mg in 50 μl aqueous DMSO) was injected intra-tumorally, ^{19}F NMR signal was readily detected at 4.7 T. Over a period of 30min conversion of OFPNPG to product OFPNP was observed unequivocally demonstrating β-gal activity (see spectra). Tumor and tissues were also examined by Western blots and β-gal assay for activity. High β-gal activity was found in the tumor, with minimal activity in normal tissues. This approach directly reveals β-gal activity, which could be used in tandem with therapeutic genes to monitor therapy. As gene therapy becomes a reality, the ability to detect transgenic expression non-invasively will become increasingly important for treatment planning and optimization. The prototype gene reporter molecule (OFPNPG) releases a potentially toxic product (fluoronitrophenol) and we are also exploring whether this can serve as the basis for broad-spectrum chemotherapy. Meanwhile, we are also developing new generations of ^{19}F NMR reporter designed to minimize toxicity.



Keywords: β-galactosidase, ^{19}F NMR, lacZ gene, prostate cancer, gene therapy
Supported by DOD PC031075 (LL), NCI pre-ICMIC CA86354 and P41-RR02584
The 97th AACR meeting, Washington DC, April, 2006

Novel "Smart" 1H MRI Contrast Agents for Assessing LacZ Gene Expression

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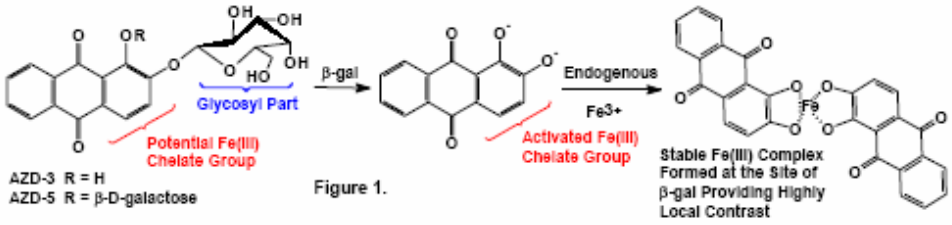
Introduction

The application of reporter genes to study gene expression and regulation in biological systems is common practice. Among the widely used reporter proteins, β-gal (*lacZ*) is recognized as the most attractive reporter gene, and its introduction has become a standard means of assaying clonal insertion, transcriptional activation, protein expression, and protein interaction. Many colorimetric substrates are available commercially, but *in vivo* assays would be more powerful. Recently, Weissleder *et al.*[1] presented a near infrared *in vivo* approach based on DDAOG, Meade *et al.*[2] reported a proton MRI approach using EgadMe, and Mason *et al.*[3,4] presented both proton and ^{19}F NMR methods using S-gal™ and fluorophenol β-D-galactosides. S-gal™ was effective, but the molecule was designed for histology and can be optimized for *in vivo* MRI applications. We now present analogs of S-gal™ further demonstrating this fundamentally novel mechanism of "smart" ^{1}H MRI contrast agent, whereby the paramagnetic material is not generated until β-gal acts on the substrates (here AZD-3 or AZD-5) in the presence of Fe^{3+} ions to generate a precipitate (Figure 1).



AZD-3 R = H
AZD-5 R = β-D-galactose

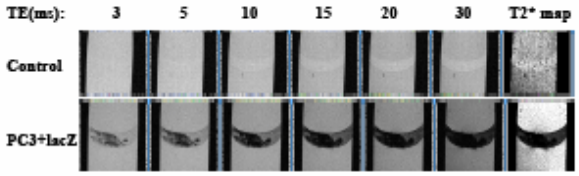
Figure 1.

Materials and Methods

AZD-3 and AZD-5 were stereoselectively synthesized and characterized in our lab. MR images were obtained using a Varian Unity INOVA 400 NMR spectrometer with gradient echo imaging: TR=100ms, Flip angle=10°, TE=multiple values 3-30ms, Matrix=256×128, FOV=48×24mm. As an example 10^5 PC3-LacZ or wild type cells were layered in agarose ferric ammonium citrate (2.5 μg/mL) and AZD-5 (1.5 μg/mL).

Results

A series of tests in solution and cultured tumor cells proved the principle. Both AZD-3 and AZD-5 were cleaved effectively by β-gal generating an intense black precipitate, which provides strong T_2^* relaxation and intense Fe(III)-based ^{1}H MRI contrast (Figure 2).



Conclusion

These results provide further evidence for the broad specificity of β-gal to cleave diverse substrates. The black paramagnetic precipitate is analogous to that formed using commercial S-gal™ and demonstrates the potential for derivatizing the substrate to optimize the MR active molecule. Here, ferric ions were added. However, it is noteworthy that tumor cells, as compared with their normal counterparts, frequently exhibit increased uptake and utilization of iron and thus endogenous ferric ions may suffice for *in vivo* applications. We believe, this novel "smart" Fe(III)-based ^{1}H MRI contrast agent mechanism holds great promise as a fundamentally different ^{1}H MRI platform for *in vivo* assessing *lacZ* gene activity.

Supported by Cancer Imaging Program P20 CA086354 and BTRP P41RR02584.

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ISMRM 13th Scientific Meeting in Miami Beach, Florida, USA, May 2005

^{19}F CSI of gene-reporter molecule OFPNPG

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Synopsis: The *lacZ* gene, encoding the enzyme β-galactosidase (β-gal) was historically the most attractive reporter gene for molecular biology. 2-Fluoro-4-nitrophenol-β-D-galactopyranoside (OFPNPG) belongs to a novel class of NMR active molecules (fluorophenyl-β-D-galactopyranosides), which are highly responsive to the action of β-gal. OFPNPG has a single ^{19}F peak with chemical shift of 55 ppm. It is cleaved by β-gal to OFPNP, which has a pH sensitive chemical shift of 59-61 ppm. The large change in the chemical shift allows us to image β-gal activity with magnetic resonance chemical shift imaging (CSI). We will present the results of ^{19}F CSI studies of enzyme activity and *lacZ* gene expression in 9L glioma and PC3 cells. Our results indicate that OFPNPG is a promising gene-reporter molecule for future *in vivo* studies.

ISMRM 13th Scientific Meeting in Miami Beach, Florida, USA, May 2005

In vivo detection of lacZ gene expression in a human prostate xenograft tumor By ^{19}F NMR CSI using OFPNPG

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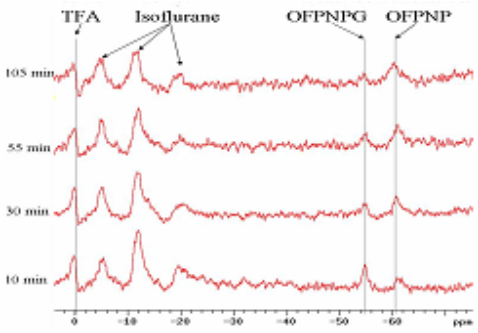
Introduction

The *lacZ* gene, encoding the enzyme β-galactosidase (β-gal), has historically been the most common reporter gene used in molecular biology. Many chromogenic or fluorogenic substrates are well established, but they are generally limited to histology or *in vitro* assays. 2-Fluoro-4-nitrophenol-β-D-galactopyranoside (OFPNPG) belongs to a novel class of NMR active molecules (fluorophenyl-β-D-galactopyranosides), which are highly responsive to the action of β-gal. OFPNPG has a single ^{19}F NMR signal with chemical shift of 55 ppm. It is cleaved by β-gal to OFPNP, which has a pH sensitive chemical shift of 59-61 ppm. The large change in the chemical shift allows us to assess β-gal activity with magnetic resonance chemical shift imaging (CSI).

Methods

PC3-lacZ tumor cells were implanted in the thigh of SCID mice and allowed to grow to about 1 cm^3. When a solution of OFPNPG (4 mg in 50 µl water:DMSO::1:1 mixture with sodium trifluoroacetate (TFA) as a chemical shift reference standard) was injected intra-tumorally, signal was readily detected using a spin-echo CSI sequence at 4.7 T. Over a period of 2 h conversion of OFPNPG to product OFPNP was revealed by development of new upfield signal unequivocally demonstrating β-gal activity (see spectra)



Conclusion

These results provide our first observations in a tumor xenograft *in vivo* and show promise for the use of OFPNPG as gene-reporter molecule for future studies. Particular virtues of the NMR approach are the ability to detect specific substrate loss accompanied by product development unequivocally revealing enzyme activity. Simultaneously other metabolites can be observed here, the chemical shift standard TFA together with signals for the anesthetic isoflurane. As gene therapy becomes a reality, the ability to detect transgene expression non-invasively will become increasingly important for treatment planning and optimization.

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Molecular Medicine Symposium, Houston, Feb. 2005